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# Molecular diagnosis of anaplasmosis in Cattle in and around Bidar (Karnataka)

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**Abstract**
Bovine anaplasmosis is one of the most prevalent tick-transmitted diseases of cattle worldwide. The present study was conducted to compare the efficacy of conservative thin blood smear (TBS) examination with molecular diagnosis using PCR for detection of Anaplasmosis. A total of 150 blood samples from all the five talukas of Bidar district were collected from animals having tick infestation and clinical signs suggestive of anaplasmosis namely anorexia, high fever, anaemia, icteric visible mucus membrane, debility and abortion for a period from November 2017 to July 2018. The blood samples were subjected to blood smear examination and PCR assay. A total of 150 DNA samples were analyzed by polymerase chain reaction out of which 42 samples were found positive for *Anaplasma marginal*e suggesting prevalence of 28 per cent. In the present investigation, sensitivity and specificity of thin blood smear examination was 40.47 per cent and 100 per cent as compared to that of the gold standard PCR with sensitivity and specificity of 100 per cent. Cent per cent sensitivity and specificity of PCR proved the superiority of molecular diagnostic test over routine thin blood smear examination.

**Keywords:** Anaplasmosis, bovine, PCR, sensitivity, specificity

## Introduction
Livestock plays an important role in Indian economy and contributes up to 4.11 per cent in national GDP. About two third of rural community (20.5 million) depends on the livestock for their livelihood through employment, food, social security and income generation (Annual report of DAHD, 2016-17) [1]. The climatic variation in the tropical and sub-tropical countries poses a threat of many infectious, metabolic and parasitic diseases in India. Among the parasitic diseases, haemoprotozoan diseases of critical importance causing not only economic loss to farmers but also indirectly affecting the nations GDP (Annual report of DAHD, 2016-17) [1].

Bovine anaplasmosis is one of the most prevalent tick-transmitted diseases of cattle worldwide (Kocan *et al*., 2003) [7] and is of greatest economic significance in cattle farming (Smith, 1996) [12]. Diagnosis of anaplasmosis often poses a challenge as clinical manifestations are inconspicuous and non-specific. *A. marginale* can be detected in Giemsa-stained thin blood smears (TBS) from clinically infected animals during the acute phase of the disease. In spite of low sensitivity, TBS is the main laboratory diagnostic method under field condition. Development of the newer technology diagnostic aids such as serological and molecular based tests have added advantage of more sensitivity and specificity. Polymerase chain reaction (PCR) based methods have been developed which are capable of detecting low levels of infection in infected as well as carrier animals. PCR assay targeted at the Anaplasma msp4 and/or msp1a genes have been used to differentiate isolates of *A. marginale* which is useful to track the origin of an outbreak, and to differentiate between different species of Anaplasma such as *A. marginale* and *A. centrale* (Fuente *et al*., 2001; Lew *et al.,* 2002) [4, 9]. The present study was conducted to compare the efficacy of conservative thin blood smear (TBS) examination with molecular diagnosis using PCR for detection of Anaplasmosis.

## Materials and Methods
A total of 150 blood samples from all the five talukas of Bidar district were collected from animals having tick infestation and clinical signs suggestive of anaplasmosis namely anorexia, high fever, anaemia, icteric visible mucus membrane, debility and abortion for a period from November 2017 to July 2018.

The blood samples were subjected to blood smear examination and PCR assay.

**Thin blood smear (TBS) examination**

Blood was collected from the ear vein to make the smear. Prepared smears were air dried and fixed with absolute methanol (5 mins), stained with 10% Giemsa stain for a time period of 30 minutes and examined under oil immersion to observe *Anaplasma sp*. organisms These rickettsial organisms were identified according to the characters described by Soulsby (1982) [13].

**Polymerase Chain Reaction Test**

**DNA Extraction**

Genomic DNA extraction was performed according to the QIAamp DNA Mini Kit as per manufacturer's instructions (Qiagen) and its quality and quantity was determined using Nano drop and gel electrophoresis. Blood samples (stored at -20$^0$ C in EDTA vacutainer tubes) were thawed, standard citrate buffer was added, mixed and the tubes were centrifuged. The top portion of the supernatant was discarded and additional buffer was added, mixed and again the tube was centrifuged. After discarding the supernatant, the pellet was resuspended in a solution of SDS detergent and proteinase K, and the mixture was incubated at 55$^0$ C for one hour in water bath. The sample then was phenol extracted once with a phenol-chloroform solution, and after centrifugation, the aqueous layer was removed to a fresh microcentrifuge tube. The DNA was ethanol precipitated, resuspended in buffer and then ethanol precipitated for second time. Once the pellet was dried, buffer was added and the DNA was resuspended by incubation at 55$^0$ C overnight. The genomic DNA solution was assayed by the polymerase chain reaction.

**PCR Analysis of samples**

The Primers for *Anaplasma marginale* used in the present study are described in Table 1. The primers for PCR were performed according to method described by Christine *et al*., (1995) [3].

**Table 1:** Oligonucleotide primers used to amplify and small subunit ribosomal RNA gene sequences of *Anaplasma marginale* primer sequence

| Primer | Primer Sequence | Expected amplification size | Source |
|---|---|---|---|
| **PRIMER SET A *Anaplasma spp*** | | | |
| Forward | TCCTGGCTCAGAACGAACGCTGGCGGC | 1433bp | (Zuoyong *et al.*, 2018) |
| Reverse | AGTCACTGACCCAACCTTAAATGGCTG | | |
| **PRIMER SET B *Anaplasma marginale*** | | | |
| Forward | CAGCTCTAGCAGGTTAGGCG | 474 bp | (George *et al.*, 2016) [5] |
| Reverse | TCATGTTCTGCGTTTCTGGG | | |
| **PRIMER SET C *Anaplasma centrale*** | | | |
| Forward | CTGCTTTTAATACTGCAGGACTA | 426bp | (Kawahara *et al.*, 2006) |
| Reverse | ATGCAGCACCTGTGTGAGGT | | |
| **PRIMER SET D *Anaplasma bovis*** | | | |
| Forward | CTCGTAGCTTGCTATGAGAAC | 551bp | (Zuoyong *et al.*, 2018) |
| Reverse | TCTCCCGGACTCCAGTCTG | | |

**Analysis of amplified product**

Analysis of amplified product was made by gel electrophoresis. The amplified product was separated by electrophoresis on a 2 per cent agarose gel.

**Results and Discussion**

**Blood smear examination**

The microscopic examination of the Giemsa-stained thin blood smears of naturally infected 12 cattle revealed the presence of round, dense dot like organisms in the vacuoles on or near margin of the infected erythrocytes morphologically compatible with *Anaplasma marginale* organism.

Seventeen blood smears examination revealed presence of dense, rounded, intra-erythrocytic bodies situated at the margin of erythrocytes among 150 blood samples screened. Similar results were observed by Singh *et al.* (2012) [11] and Kumar *et al.* (2015) [8] in dairy cattle.

**Polymerase chain reaction**

A total of 150 DNA samples were analyzed by polymerase chain reaction out of which 42 samples were found positive for *Anaplasma marginal*e suggesting prevalence of 28 per cent. 474 bp fragment of *A. marginale* msp4 gene was amplified and run on 2 per cent gel electrophoresis. Similar gene amplification was done by Picoloto *et al.* (2010) [10] and George *et al.* (2016) [5] to identify *Anaplasma marginale* organisms. The pictorial images of gel electrophoresis are shown in Fig. 1 for *Anaplasma spp* and in Fig. 2 for *Anaplasma marginale*.

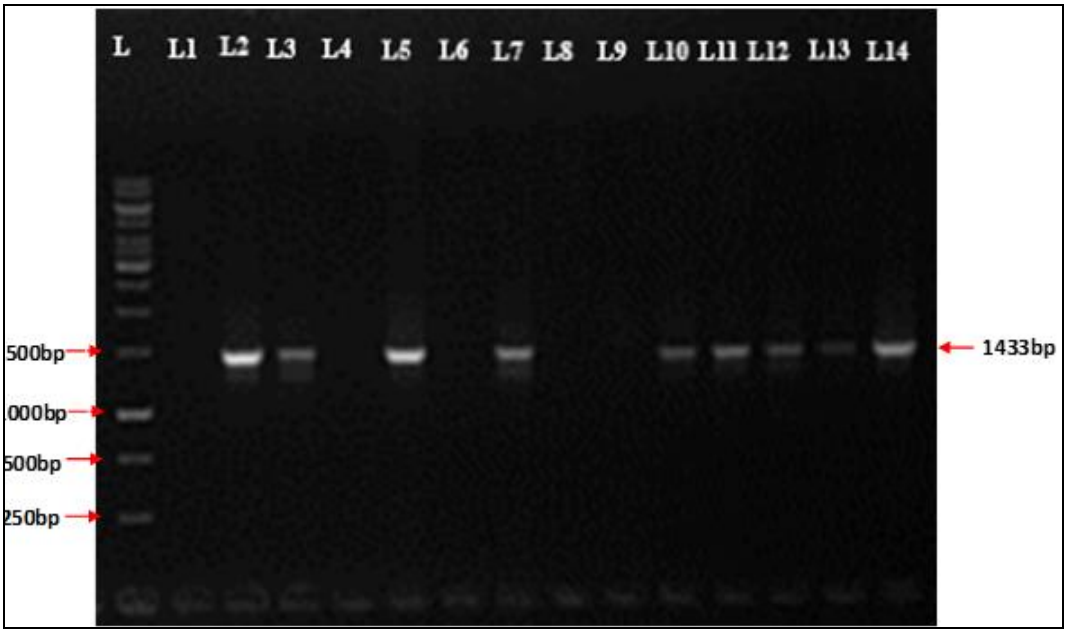
**Efficacy of Blood smear examination over PCR**

Diagnostic efficiency of a laboratory test was measured on the scale of sensitivity and specificity. In the present investigation sensitivity and specificity of direct blood smear examination over molecular diagnostic test was done (Table 2).

In the present investigation, sensitivity and specificity of thin blood smear examination was 40.47 per cent and 100 per cent as compared to that of the gold standard PCR with sensitivity and specificity of 100 per cent. Cent per cent sensitivity and specificity of PCR proved the superiority of molecular diagnostic test over routine thin blood smear examination. The results of present investigation are in line with reports of Jaswal *et al.* (2014) [6] and Ashker *et al.* (2015) [2] in tick infected dairy cows. However, though thin blood smear exam was less sensitive (40.47%), 100 per cent specificity suggested that it can be used as primary laboratory test for diagnosis of anaplasmosis under field conditions.

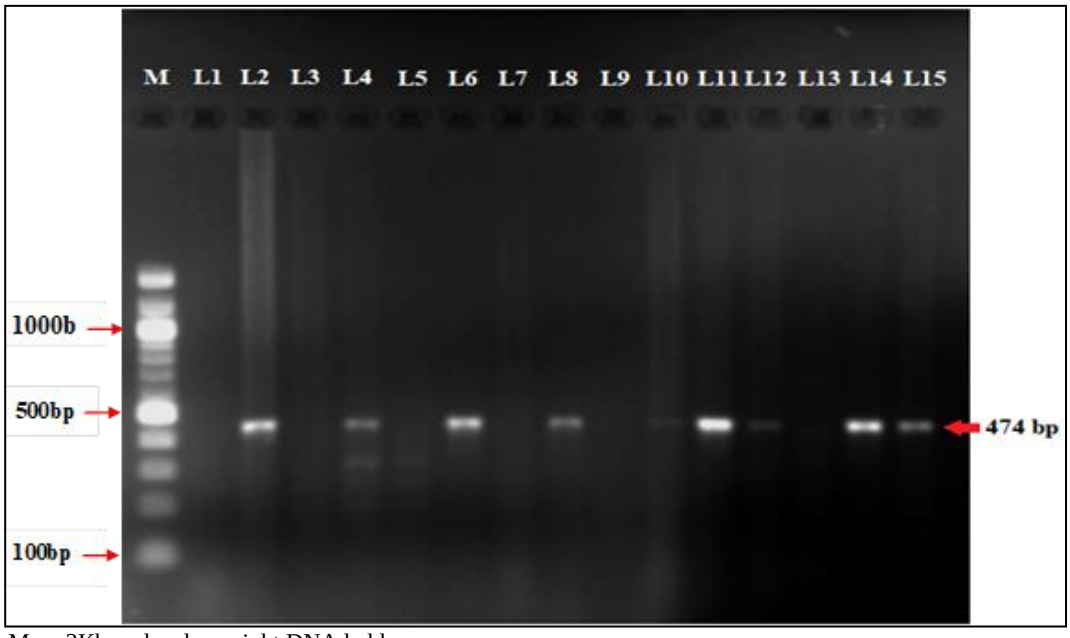
**Table 2:** Sensitivity and Specificity of Blood smear examination

| Screening test result (Blood smear examination) | True status PCR | | Total |
|---|---|---|---|
| | Diseased | Non- Diseased | |
| Positive | 17 | 0 | 17 |
| Negative | 25 | 108 | 133 |
| Total | 42 | 108 | 150 |




L --- DNA ladder
L1 --- Negative control
L2 --- Positive control
L4, L6, L8, L10, L11, L12, L14 and L15-- Positive for *Anaplasma marginale*

**Fig 1:** Analysis of amplified product (1433 bp) from blood samples with primers set A (*Anaplasma Spp*)




M --- 3Kb molecular weight DNA ladder
L1 --- Negative control
L2 --- Positive control
L4, L6, L8, L10, L11, L12, L14 and L15-- Positive for *Anaplasma marginale*

**Fig 2:** Analysis of amplified product (474 bp) from blood samples with primers set B (*Anaplasma marginale*)

## Conclusion

Cent per cent sensitivity and specificity of PCR proved the superiority of molecular diagnostic test over routine thin blood smear examination against bovine anaplasmosis.

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